Neuroproteomics and the Detection of Regulatory Phosphosites

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Abstract: Protein phosphorylation is a key post-translational modification that controls intracellular signalling in virtually all cell types. In the nervous system, it contributes to the regulation of neuronal signalling and control processes underlying synaptic plasticity and cognitive functions. However, despite its importance, knowledge about phosphoproteins and their phosphosites in the brain remains limited. A pre-requisite for unravelling brain biology and function at the molecular level, are the qualitative and quantitative analyses of protein phosphorylation and its dynamics. These analyses of the phosphoproteome require novel methodologies in addition to traditional biochemical methods. Current phosphoproteomic workflows have reached a level of maturity, which allow for their use in combination with molecular approaches, and their application to the study of higher order brain function and cognitive processes. Neuroproteomics is emerging as an essential new sub-field of the neurosciences. This review focuses on the recent advances in the application of neuroproteomics to the phosphoproteome and discusses the challenges to come.

Key Words: Phosphoproteomics, phosphorylation, phosphopeptide enrichment, stable isotope labelling, quantitative mass spectrometry, neuroproteomics, nerve cell/neuron.

INTRODUCTION TO PROTEIN PHOSPHORYLATION IN THE CENTRAL NERVOUS SYSTEM

Phosphorylation and Dephosphorylation of Synaptic Proteins

Phosphorylation is the most common and important form of reversible protein post-translational modification (PTM), with up to 30 % of all proteins being phosphorylated at any given time (Hunter, 1998). Protein kinases are the effectors of phosphorylation, and catalyse the transfer of the γ-phosphate from ATP to specific amino acids on proteins. They generally belong to a single super-family containing a eukaryotic protein kinase catalytic domain (Ubersax and Ferrell, 2007). Several hundred protein kinases exist in mammals and are classified into distinct superfamilies. Proteins are phosphorylated predominantly on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues, with each accounting for approximately 86, 12 and 2 % respectively of the human phosphoproteome (Olsen *et al.,* 2006). Protein phosphatases, the primary effectors of dephosphorylation, can be grouped into three main classes based on sequence, structure and catalytic function. The main and largest class includes the phosphoprotein phosphatase (PPP) family with PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7, and the protein phosphatase Mg^{2+}- or Mn^{2+}-dependent (PPM) family, with PP2C. The protein Tyr phosphatases (PTP) super-family forms the second group, and the aspartate-based protein phosphatases the third (Moorhead *et al.,* 2007).

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Protein phosphorylation plays a crucial role in biological functions and controls nearly every cellular process (see Fig. **1**), including metabolism, gene transcription and translation, cell-cycle progression, cytoskeletal rearrangement, protein-protein interactions, protein stability, cell movement, and apoptosis (Manning *et al.,* 2002; Ubersax and Ferrell, 2007). These processes depend on the highly regulated and opposing action of protein kinases and phosphatases, through changes in the phosphorylation of key proteins (Blitzer *et al.,* 2005). Phosphorylation, along with methylation and acetylation, also regulates access to DNA through the covalent modification of histone proteins (Jenuwein and Allis, 2001).

One of the major switches for neuronal activity is the activation of protein kinases and phosphatases by elevation of intracellular calcium. The degree of activation of the various isoforms of protein kinases and phosphatases is controlled by their differential sensitivity to calcium (Lee, 2006). Furthermore, a wide range of specific inhibitors and targeting partners such as scaffolding, anchoring, and adaptor proteins also contribute to the control of kinases and phosphatases and recruit them into signalling complexes in neuronal cells (Sim and Scott, 1999). Such signalling complexes typically act to bring kinases and phosphatases in close proximity with target substrates and signalling molecules, and enhance their selectivity by favouring their accessibility to these substrate proteins (Faux and Scott, 1996). Phosphorylation events, therefore, are controlled not only by the balanced activity of protein kinases and phosphatases, but also by their restricted localisation in the cell. These regulators are essential for maintaining the coordinated action of signalling cascades, including short-term (synaptic) and long-term (nuclear) signalling.

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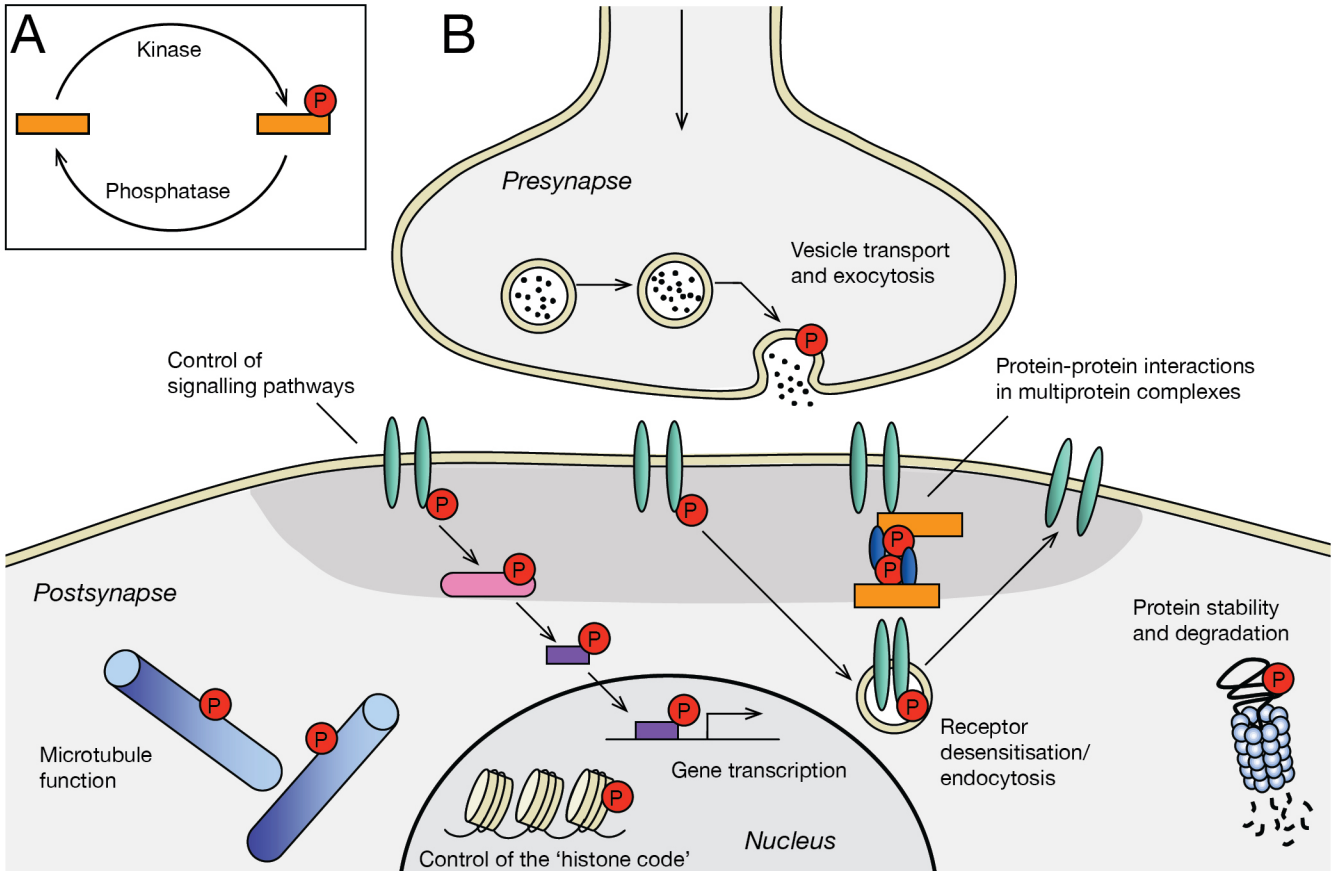

Fig. (1). Role of phosphorylation in the synapse.

A). The phosphorylation state of a protein is determined by the competing actions of phosphatases and kinases. **B**). In the neuron, phosphorylation is involved in a diverse range of cellular activities, including vesicle transport and exocytosis, cellular signalling, protein-protein interactions, protein stability and degradation, receptor desensitisation and endocytosis, microtubule function, gene transcription and control of the histone code.

Protein Phosphorylation in Higher Order Brain Functions

In the brain, protein phosphorylation controls neuronal signalling and glial cell function. Additionally, it is an important regulator of the coordinated activity of neuronal networks. Concomitant change in the activity of individual neurons and their pre- and post-synaptic partners is the basis of the transmission of information through neuronal circuits. Thus, the acquisition and the storage of information requires specific and long-lasting changes in the strength of individual synaptic contacts, a property of neuronal cells defined as synaptic plasticity. The dominant model of activity-dependent synaptic plasticity in the adult brain is long-term potentiation (LTP). The induction of most forms of LTP depends on the activation of NMDA receptors by depolarisation of the post-synaptic cell, and a rise in intracellular calcium. This results in the activation of multiple calcium-dependent enzymes, in particular, the calcium/calmodulin-dependent protein kinase (CaMKII), which then autophosphorylates and therefore remains active even after calcium returns to basal levels. The subsequent phosphorylation of the AMPA receptor subunit GluR1, by CaMKII, favours the recruitment of new receptors to the membrane and increases the channel's conductance, resulting in long-lasting functional changes (Malenka and Nicoll, 1999). Although some pathways regulated by phosphorylation have been delineated, a global picture of phosphorylation-dependent changes in the brain is lacking, due in part to the traditional lack of suitable technologies to measure protein phosphorylation.

THE BENEFITS OF MASS SPECTROMETRY FOR THE ANALYSIS OF PHOSPHORYLATION SITES

The advent of proteomics has opened a new dimension to genomics, and has provided novel information about gene expression at the protein level. Its importance is underscored by the recognition that the abundance of mRNA transcripts and the corresponding protein products often do not correlate. This is primarily due to the post-transcriptional regulation of mRNA (Griffin *et al.,* 2002). In addition to the amount, activity, localisation, and turn-over, proteins are themselves regulated by post-translational mechanisms—in particular protein phosphorylation. To understand these mechanisms, it is essential to identify and locate the residues that are controlled by (de)phosphorylation, and determine the temporal dynamics, kinetics and stoichiometry of their modification. To date, much of the knowledge gathered about phosphorylation in nerve cells was obtained by studies at the level of single molecules. However, novel proteomic methods are beginning to allow more global characterisations of phosphorylation, and analyses of its dynamics (Olsen *et al.,*

2006). Mass spectrometry is ideally suited to the determination of phosphorylation, because the addition of a phosphate group (HPO_3) to the amino acids Ser/Thr/Tyr leads to an increase of 80 daltons (Da) of the molecular mass of that residue (see Fig. **2**). This allows the phosphorylation site to be assigned through the observation of a discrete mass increment of the peptide in the mass spectrometer (MS) scan (peptide mass), or of the specific Ser/Thr/Tyr residue in the MS/MS scan (peptide fragmentation pattern) (Steen and Mann, 2004). Mass spectrometry, therefore, allows site-specific and *de novo* assignment of phosphorylation at the level of the individual amino acid. In traditional proteomic experiments the detection of a single peptide can be sufficient to determine that a specific protein is in the sample. However, to map every phosphorylation site of a protein requires (in theory) the detection of every proteolytic peptide derived from that protein. At present, this is an unfeasible proposition in a typical shotgun proteomic experiment.

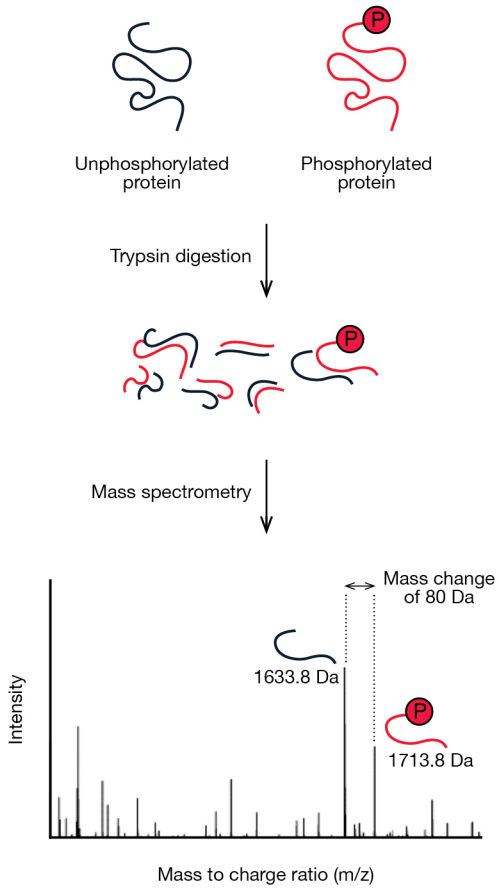

Fig. (2). Determining phosphorylation sites using mass spectrometry.

The detection of phosphorylation by mass spectrometry relies on the observation of mass shifts, relative to the molecular mass of the corresponding unphosphorylated peptide. This figure shows the MS analysis of the proteolytic peptides derived from two copies of a protein, one being phosphorylated (red) and the other unphosphorylated (black). The mass difference between the phosphorylated (1713.8 Da) and unphosphorylated (1633.8 Da) forms of the modified peptide in the MS scan is 80 Da, corresponding to the presence of an HPO_3 group.

It has become evident that modified sites in proteins act together to modulate molecular interactions and protein activity. Hence, research necessitates the investigation of signalling networks in a high-throughput manner, and not single proteins in isolation. Mass spectrometry is currently the only technique amenable to large-scale, high-throughput and discovery-driven analyses. Traditional methods such as Western blotting are not suitable for the discovery of novel PTMs of proteins. Although very sensitive, Western blotting requires prior knowledge of the modification type and site, and depends on the availability of PTM-specific antibodies. Such antibodies are often problematic with sub-optimal specificity, and potential cross-reactivity to other sites on the same or different proteins. The analysis of PTMs using antibodies is often hindered by epitope occlusion by neighbouring PTMs within or around the antibody's recognised sequence (Garcia *et al.,* 2007b). Antibody-based techniques are also not suitable for high-throughput studies and quantitative analyses are challenging. These difficulties significantly increase lag-times between the discovery of a site and the generation of an antibody, which tremendously slows research.

STRATEGIES FOR THE ENRICHMENT OF PHOSPHOPEPTIDES

Because of the sub-stoichiometric nature of phosphorylation, the majority of isolated proteins are unphosphorylated. The proteolytic digests of these proteins are, therefore, dominated by unphosphorylated peptides. When analysing such samples, the ion signals of unphosphorylated peptides dominate the MS spectrum and prevent the identification of phosphorylated peptides by conventional mass spectrometry strategies. To overcome this limitation, phosphopeptide enrichment is an essential first-step in any MS analysis. Methods for the enrichment of phosphopeptides are diverse and include techniques based on specific anti-phospho antibodies such as anti-phosphorylated-Tyr (anti-pTyr), ionic interaction such as strong cationic exchange HPLC (SCX-HPLC), immobilised metal affinity chromatography (IMAC) and titanium-dioxide (TiO_2) affinity enrichment, or the chemical derivatisation of phosphopeptides such as phosphoramidate chemistry (PAC). While the outcome of all methods is a less complex sample in which phosphopeptides are the dominant species, each method results in a qualitatively different end-sample. The following section will provide an overview of these methods and outline some of their advantages and drawbacks.

Antibody Based Enrichment of Phosphopeptides

Phosphorylation at Ser or Thr is more common than phosphorylation at Tyr residues. A consequence of this is that studies using the general enrichment methods described below only reported 2-4% of sites as pTyr (Munton *et al.,* 2007; Olsen *et al.,* 2006). Therefore, to investigate pTyr, it is necessary to use enrichment methods that specifically isolate these sites, such as pTyr antibodies. A compelling reason to use antibodies is that one specific type of phosphorylation, such as pTyr (Rush *et al.,* 2005) or alternatively phosphorylated Ser/Thr (pSer/pThr) (Gronborg *et al.*, 2002) can be isolated. The selective enrichment of pTyr using antibodies, can also be used in combination with the more general enrichment methods, IMAC, TiO_2 or SCX-HPLC.

Ionic Affinity Interaction Based Enrichment of Phosphopeptides

IMAC takes advantage of the affinity of the negatively charged phosphate group for trivalent ions, typically Fe^{3+} (Stensballe *et al.,* 2001; Tao *et al.*, 2007) or Ga^{3+} (Posewitz and Tempst, 1999). Its specificity is improved by the conversion of the carboxylic groups of peptides to the corresponding methyl-esters which reduces non-specific binding of unphosphorylated peptides to the IMAC affinity matrix (Ficarro *et al.*, 2002). While IMAC is typically used to enrich phosphopeptides from digested samples, the method has also been useful for the enrichment of intact phosphoproteins (Collins *et al.,* 2005). A similar technique, which makes use of TiO_2-based solid-phase material, has been shown to bind phosphopeptides very selectively (Pinkse *et al.,* 2004; Thingholm *et al.,* 2006). Phosphopeptide specificity is further improved by the addition of DHB (2,5-dihydroxyenzoic acid) to the binding solution (Larsen *et al.,* 2005).

SCX-HPLC separates peptides/proteins based on solution-state-charge, which results from the protonation and deprotonation of basic and acid groups. Phosphorylation reduces the charge state of peptides at low pH allowing the phosphopeptides to be enriched as they elute earlier than other peptides from the SCX column (Beausoleil *et al.,* 2004). SCX-HPLC has the added advantage of separating the peptide mixture into smaller and simpler fractions (Peng *et al.*, 2003), but also results in greater loss due to additional processing, and is only suitable for tryptic peptides (Ballif *et al.,* 2004). However, it is a powerful method that can be utilised upstream of other enrichment methods, such as IMAC (Gruhler *et al.,* 2005; Trinidad *et al.,* 2006; Munton *et al.,* 2007).

Chemical Derivatisation and Enrichment of Phosphopeptides

Chemical derivatisation methods are based on the chemical modification of the phosphate group to facilitate isolation of the phosphopeptide. In the β-elimination strategy phosphate groups are removed and then replaced with an affinity group by Michael addition. The phosphorylated peptides can later be captured using the affinity group (Oda *et al.,* 2001; Jaffe *et al.*, 1998). An alternative strategy is based on the PAC method, in which phosphopetides are chemically derivatised by methyl esterification and covalently bound to a dendrimer using a phosphoramidate linkage (Tao *et al.,* 2005; Zhou *et al.*, 2001; Tao *et al.,* 2007). In both cases, the phosphopeptides are covalently bound to a solid-phase support, allowing for harsher washing steps in order to obtain a sample more highly enriched in phosphopeptides. The PAC procedure also allows for the addition of a mass tag during the methyl-esterification reaction, which can be used for relative peptide quantification strategies (see later). Both procedures are significantly more complicated than IMAC or TiO_2, and in general require larger amounts of starting material, due to the sample loss from additional steps in the purification process. A drawback of the β-elimination method is the incompatibility with pTyr analysis, and the fact that side-reactions can lead to unphosphorylated Ser being tagged (McLachlin and Chait, 2003).

For all the phosphopeptide enrichment methods discussed above, sample clean-up steps are critical prior to enrichment. Acetone precipitation prior to, or C18 purification after proteolytic digestion, removes abundant phosphorylated species, such as lipids and DNA that would otherwise hinder the enrichment of phosphorylated peptides. Each of the described phosphopeptide enrichment strategies also has its advantages and disadvantages, depending on the biological question asked. At present, in order to globally profile phosphorylation sites in a discovery-driven experimental workflow, a combination of the described strategies (Bodenmiller *et al.,* 2007) in addition to different MS platforms and search engines (Elias *et al.,* 2005), is recommended. In turn, for answering a specific question at hand, workflows can be developed by using the available 'proteomic modules' in a targeted strategy.

STRATEGIES FOR THE RELATIVE AND ABSOLUTE QUANTIFICATION OF PHOSPHORYLATION

There are two types of quantitative data in mass spectrometry experiments: the relative change in a protein between two samples, and the absolute amount of a protein in a sample. In relative quantification the amount of a given protein is defined in relation to another measure of the same protein, such as the relative difference in protein abundance between different strains of mice. When the absolute amount of a protein is known (e.g. in picomoles), relative comparisons can also be made. Quantification can take place in the MS scan, or in the MS/MS scan, and is based on a comparison between the signal intensity of two or more peaks corresponding to the same peptide from different treatments (see Fig. **3B**). Mass spectrometry is not considered an inherently quantitative technique, mainly because the efficiency with which peptides ionise and enter the MS depends on both their composition and the local chemical environment (Steen and Pandey, 2002). In practice, this means that the signal intensities of different peptides from the same protein are very different. However, for the same peptide under the same experimental conditions and MS analysis, the signal intensity is linearly related to the sample amount.

To perform a comparative study between mice or differently treated cells, samples must be combined prior to MS analysis. To achieve this while still retaining information about the origin of each detected peptide, differential labelling strategies must be used. Labelling introduces a differential mass tag into the peptide, allowing two or more experimental samples to be combined and analysed together, and allows a given peptide to be assigned to its source. Labelling is achieved either chemically (iTRAQ, methyl-esterification, propionylation, ICAT), metabolically (SILAC, SILAM) or enzymatically (^{18}O) and is performed at the protein or peptide level (see below). The comparative analysis of protein modifications such as phosphorylation, is complicated by the fact that phosphorylation must also be considered in the context of changes in the amount of the protein itself. Hence, the ratio of both the phosphorylated peptide and its unphosphorylated counterpart must be determined to ascertain whether changes in phosphorylation are not simply due to a difference in the overall amount of the protein. Quantification, therefore, must take place at the level of the phospho-

peptide relative to the level of unphosphorylated peptides from the same protein.

CHEMICAL LABELLING OF PHOSPHOPEPTIDES FOR QUANTITATIVE STUDIES

The chemical addition of a mass tag to a peptide/protein is a method to allow comparative studies. Chemical labelling strategies have the advantage that any tissue can be labelled, and can therefore be added to existing proteomic studies without the need to change the overall workflow. A disadvantage of this technique is that more peptide/protein is required in order to reach the same sensitivity compared to unlabelled MS experiments, due to additional sample handling steps and the associated losses. Several different approaches can be utilised, and are discussed in the following sections.

iTRAQ Labelling of Phosphopeptides

The iTRAQ (isobaric tags for relative and absolute quantification) tag is a labelling reagent, which currently allows the comparison of up to four different samples in a single experiment (see Fig. **3**); an 8-plex version is also under development. iTRAQ is based on the differential covalent labelling of peptides (or proteins (Enoksson *et al.,* 2007)) with one of four iTRAQ reagents resulting in the incorporation of 144.1 Da to the peptide's primary amines within the N-termini and lysine residues. The iTRAQ tag incorporates a balance group such that peptides with different tags are indistinguishable by mass. However, they can be differentiated upon peptide fragmentation by collision induced fragmentation (CID) through the release of reporter ions, each of which has a different mass (114.1, 115.1, 116.1, or 117.1 Da) (see Fig. **3B**). Comparison of the intensity of the reporter ions allows the relative quantification of labelled peptides. Finally, two points regarding the use of iTRAQ should be noted. Because of the low mass of the reporter ions in the MS/MS scan, not all machines are currently suitable for iTRAQ experiments, due to the fact that many cannot measure the low molecular weight mass range consistently. Also, phosphopeptides elute earlier than non-phosphopeptides on SCX-, RP-HPLC and C18. Therefore, special care should also be taken during the C18/SCX cleanup after iTRAQ labelling in order to avoid losing them.

Methyl-Esterification/Propionylation of Phosphopeptides

Two similar methods for the introduction of a mass difference are methyl-esterification and propionylation. In addition to improving the specificity of IMAC, methyl-esterification of N-termini and carboxylic groups using either d0 or d4 methanol introduces a mass difference of 3 Da between samples (Goodlett *et al.,* 2001). The propionylation of N-termini and lysine residues using either d0 or d5 propionic anhydride also results in a mass difference of 5 Da between the two samples (Jin *et al.,* 2005). Both methods have the advantage of being extremely cheap, allowing the use of large amounts of starting material, but do involve complex chemistry. The more complicated handling inherent in both techniques makes their application in neuroproteomics difficult, due to the typically small amount of starting tissue in CNS studies.

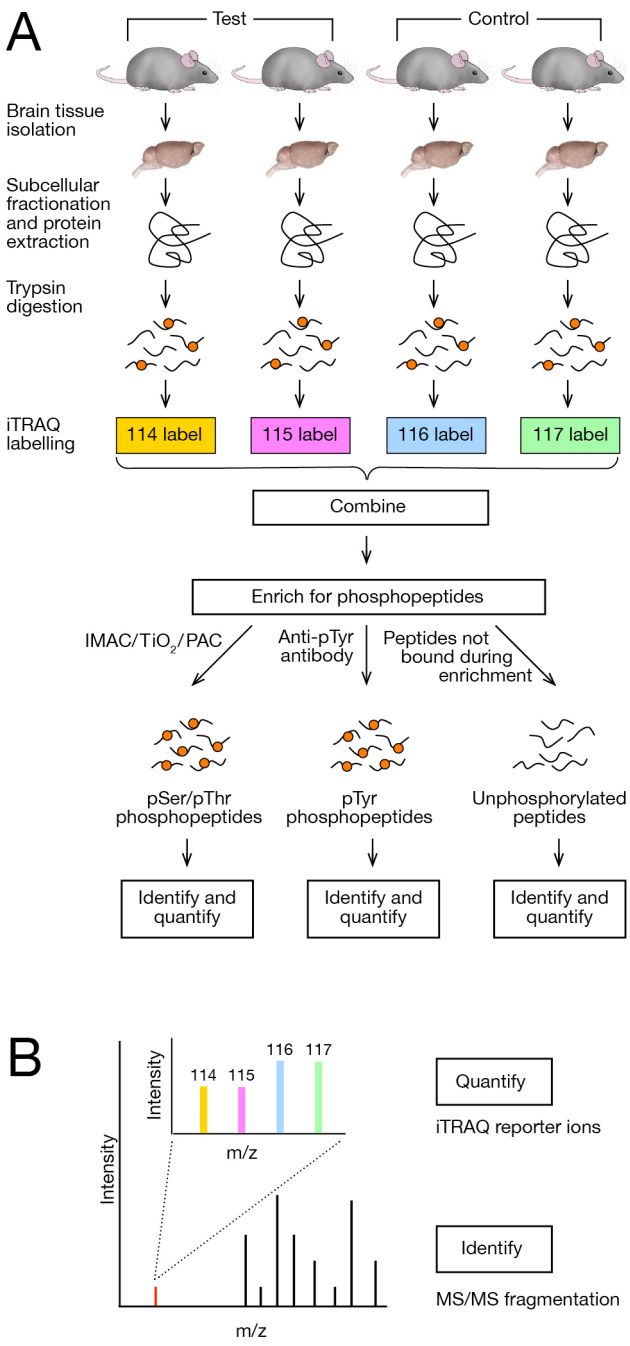

Fig. (3). iTRAQ workflow for the analysis of pSer/pThr and pTyr sites.

iTRAQ in combination with phosphopeptide enrichment allows the relative quantification of peptides and phosphopeptides. **A**) Schematic workflow for the isolation, identification, and quantification using iTRAQ, of pThr/pSer and pTyr phosphopeptides along with unphosphorylated peptides. **B**) Peptide identification and the determination of the phosphorylated residue takes place in the MS/MS scan. Quantification is possible by comparing the peak intensities of the iTRAQ reporter ions.

ICAT Labelling of Phosphopeptides

ICAT (Isotope coded affinity tags, third generation) is a protein labelling reagent that allows two samples to be com-

pared. The mass tag exists in a heavy and light form and targets the cysteine residues of proteins (Shiio and Aebersold, 2006). The fact that only cysteine-containing peptides are mass tag labelled and isolated during the ICAT procedure has the advantage of reducing sample complexity, but at the same time has the downside that phosphopeptides without cysteines will be lost. For this reason, the method has had limited application to phosphoproteomic studies.

Metabolic Labelling of Phosphopeptides

SILAC (stable isotope labelling of amino acids in cell culture) is based on growing cell lines in a medium (SILAC medium) in which a standard essential amino acid is replaced with a non-radioactive, isotopically labelled form of that amino acid. Typically the amino acids replaced are arginine and/or lysine ($^{12}C/^{13}C$, $^{14}N/^{15}N$). After several cell divisions, nearly all proteins in the cell should contain the labelled form/s of the amino acid. Proteins derived from the cells grown in the SILAC medium consequently have an increased mass in comparison to proteins derived from cells grown in normal medium and can therefore be distinguished (Ong *et al.,* 2002). By using different combinations of isotopically labelled amino acids in the SILAC medium it is also possible to compare more than two different experimental conditions. One limitation of the method, however, is that SILAC requires dividing cell lines, as the labelled amino acids are only incorporated into newly synthesised proteins. Consequently, the method cannot be used on non-dividing primary cells such as neurons. It has nonetheless been used to analyse the secreted proteome (which is composed of newly synthesised proteins) of cultured non-dividing primary cells (Gronborg *et al.,* 2006; An *et al.,* 2006), and works well with dividing cells from the CNS such as glia (McLaughlin *et al.,* 2006; Zhou *et al.,* 2005). SILAM (stable isotope labelling of amino acids in mammals) is a promising new method, which may provide an *in vivo* equivalent to SILAC. The method is based on feeding animals with food containing heavy labelled isotopes of several amino acids. It has been shown to achieve 94 % labelling of proteins throughout all tissues of the rat, including tissues of slow protein turnover such as the brain (McClatchy *et al.*, 2007a). Unlike SILAC which is now a well established and user-friendly technique with several step-by-step protocols (Ong and Mann, 2006), SILAM still requires much more development to reach such a point. While a potentially powerful method, at present its application is limited due to the prohibitive expense and the need to feed animals over several generations. SILAC and SILAM allow near complete labelling of all proteins in the cell and are both extremely simple, requiring no extra steps after protein isolation. SILAC medium in combination with the required dialysed foetal bovine serum (FBS) is relatively expensive, and it should be noted that some cell systems may change their growth behaviour, as a result of growth factors lost during the FBS dialysation process. Regardless, the method is one of the most elegant ways of labelling protein samples, and phosphorylated peptides are less easily lost because of the simplified processing workflow.

Enzymatic Labelling of Phosphopeptides

Enzymatic strategies make use of the incorporation of two oxygen atoms into a peptide during trypsin digestion. Digesting one sample in ^{18}O and the other in ^{16}O water is a simple strategy to generate a 4 Da mass difference between the two samples. The major advantage of this technique is that labelling takes place during digestion of a sample and therefore no additional labelling or processing steps are required. However, a major drawback is that labelling is reversible and in the presence of ^{16}O water, the ^{18}O labelled sample can revert back to ^{16}O, complicating analysis. Importantly, a high mass accuracy MS is required when utilising this method, as the 4 Da mass difference will be hard to distinguish when analysing peptides of 2+ and 3+ charge states.

Label-Free Quantification of Phosphopeptides

Several label-free methods exist for relative peptide quantification. APEX (absolute protein expression profiling) based on peptide counting (Lu *et al.*, 2007), and the measurement of spectral peak intensities (Bondarenko *et al.*, 2002), are two methods that have both been applied to comparative protein expression studies. Another method is based on analysing the ion currents of phosphopeptides and their unphosphorylated counterpart to determine phosphorylation stoichiometry (Steen *et al.*, 2005). Such methods potentially provide a comparatively cheaper and simpler method for the quantification of samples, since no chemical labelling is unnecessary. Currently, stable isotope methods are thought to be more accurate, as they are less susceptible to variations between the several MS runs required for label-free MS quantification (Ong and Mann, 2006). Additionally, applying such techniques to the analysis of phosphopeptides is still in its infancy, and at present methods based on stable isotope labelling offer considerably more mature tools for quantitative studies.

Common Issues

The increase in sample complexity is one of the major issues with methods such as methyl-esterification, propionylation, ^{18}O labelling and SILAC. Modified peptides exist in both an isotopically heavy and light form, as well as other variants due to incomplete and side-reactions. Abundant peptides that exist in several isoforms are also likely to hinder detection of low abundance peptides by being preferentially selected for MS/MS analysis, reducing the overall number of unique peptides identified. This may not pose a significant problem in simple samples; however, in complex samples derived from tissues such as the brain this becomes a major stumbling block. In addition, the co-elution of peptides from the chromatographic column can lead to difficulties in quantification because the observed signal may correspond to more than a single peptide. Each method has its advantages, drawbacks and costs; an important consideration for the researcher is also the decision at which point and hence with which method to label a sample.

ABSOLUTE QUANTIFICATION OF PHOSPHOPEPTIDES

Regardless of the labelling strategy, the strategies discussed above only provide quantitative information relative to another sample. Often it is useful to also know the exact molar amount of a given peptide, or a specific phosphorylation site. To achieve absolute quantification a known amount of a labelled synthetic peptide, designed to exactly mimic the

native counterpart/s after proteolysis must be 'spiked' into the sample. By comparing the signal intensity of the labelled synthetic peptide to that of the endogenous peptide, the absolute amount of the peptide can be determined. The synthetic peptide can be generated with an isotopic mass difference as in the absolute quantification (AQUA) strategy (Gerber *et al.*, 2003), or can be labelled chemically with a tag such as iTRAQ (Munton *et al.*, 2007). The AQUA strategy has the advantage that no further labelling is necessary, the synthetic peptides can be added to the sample directly and can be differentiated from the endogenous version by a known mass difference. Labelling with iTRAQ has the advantage that three other samples can be compared, and since all peptides maintain the same mass, spiked-in synthetic peptides also enable better detection of the corresponding endogenous peptides by raising the overall signal intensity. Problematically, synthetic peptides, in particular phosphorylated ones, are expensive and often difficult or impossible to synthesise. Also a synthetic peptide is required for every single peptide or phosphorylated peptide you want to quantify. Peptides with many possible modifications and phosphorylation sites are likely to be difficult to analyse with this approach, as it is not feasible or cost-effective to generate all possible variants. As a consequence this approach has not yet been applied other than in small-scale targeted studies. A potentially simpler biological method for the generation of unphosphorylated synthetic peptides is the QconCAT strategy (Pratt *et al.*, 2006; Rivers *et al.*, 2007). In this system artificial proteins comprised of concatenated proteotypic peptides under study are expressed in *E. coli*. The protein can then be purified and proteolysed to generate peptides that can be added to a sample. A future variant of the system in which phosphorylated peptides could be produced would be a valuable resource. The creation of reference peptide banks containing previously synthesised phosphopeptides could in the future aid research in this area, and has indeed been mooted by researchers in the past.

BIOINFORMATIC VALIDATION OF PHOSPHORYLATION SITES

Unlike traditional MS based methods for peptide identification, phosphoproteomic studies require tailored bioinformatic tools to ensure high-quality data. An important consideration in this respect is that filtering strategies aimed at reducing false positives are less effective on phosphoproteomic data sets (Beausoleil *et al.*, 2006). Most scoring algorithms infer confidence based on the observation of several different peptides from the same protein, raising problems in phosphopeptide analyses. In many MS experiments it is usual to identify only a single phosphopeptide for a given protein; many proteins are phosphorylated at a single site. The site of phosphorylation can also sometimes be ambiguous. Often, several potential phosphorylation sites can be found in close proximity. In these cases specific diagnostic ions are needed to confirm a site, and can cause difficulties or prevent determination if absent. Difficulties in phosphorylation site assignment also result from the labile nature of the phosphorylation group. The release of the phosphate group as a neutral species (H_3PO_4) results in the observation of an intense 'neutral loss' peak in the MS/MS spectrum (Martin *et al.*, 2005), which can obscure peptide sequence information. This is predominantly an issue with pThr and pSer sites, as the pTyr phosphate bond is relatively stable. MS/MS/MS (MS^3) using the ion showing the loss of a phosphate group as the parent ion for further fragmentation has been one attempt to generate additional sequence information to help confirm phosphorylation site placement. This has been met with varying success: some studies have reported it as useful (Lee *et al.*, 2007), while others found it provided little additional data (Beausoleil *et al.*, 2006). Several modified scoring schema have also been proposed in attempts to improve phosphopeptide scoring: these include the PTM localisation score (Olsen *et al.*, 2006), the Ascore (Beausoleil *et al.*, 2006), and additional suggested scoring criteria (Rush *et al.*, 2005). Several studies have also attempted to increase confidence in identifications by removing phosphates after, or during phosphopeptide enrichment (Ishihama *et al.*, 2007; Torres *et al.*, 2005). However, such methods significantly increase sample handling and associated losses, and the application of such a method in one study resulted in fewer phosphopeptide identifications (Tomita *et al.*, 2005). Bioinformatics specific for the needs of phosphoproteomics is clearly an area where future work will be needed.

SUBPROTEOMIC FRACTIONATION OF BRAIN TISSUE

The field of neuroproteomics faces unique challenges resulting from the complex cellular and sub-cellular structure of the central nervous system. The brain is a functionally heterogeneous organ that has different regions with distinct and specific functions. Several thousand cell types can be distinguished on the basis of function, shape, connectivity, and the neurotransmitter/s synthesised and released (Masland, 2004). Because of this complexity, it is not biologically meaningful to apply proteomics at the level of the whole brain. A major challenge is that unlike cell culture, the starting point is not a homogenous and uniformly stimulated population of cells, meaning it is less clear where a given 'signal' is coming from. Also, most proteins are involved in a diverse range of different signalling pathways, and subsequently only a portion of the total cellular pool of a protein will be phosphorylated at a given time. It is therefore necessary to select a specific brain area and sub-fractionate the collected brain extracts to address the complexity and cellular heterogeneity of the brain. Fractionation at the sub-cellular level consists of isolating specific sub-proteomes, such as organelles (Yates *et al.*, 2005), or groups of proteins that share functional context. Robust and reproducible techniques exist for the isolation of neuronal organelles: the nucleus, mitochondria and neuron-specific areas such as the post-synaptic density (PSD), synaptic membranes and synaptic vesicles (Phillips *et al.,* 2001) (see Fig. **4**). Alternatively affinity pull-down approaches or immunoprecipitation can be used to isolate subsets of proteins, for example protein complexes, or ones that exhibit specific PTMs. Sub-cellular fractionation has the added benefit of enabling the enrichment of lower-abundance proteins, such as signalling molecules by allowing higher starting material than whole cell MS analyses. In all sample preparation procedures for phosphoproteomic studies, an important consideration is that proteins phosphorylated in response to an external stimuli are usually rapidly dephosphorylated back to the resting state

once the stimuli has been removed (Steen *et al.,* 2006), necessitating rapid extraction and processing of tissue. It is also essential to include a phosphatase and protease inhibitor cocktail mix when preparing samples to prevent protein degradation or the loss of the phosphorylation.

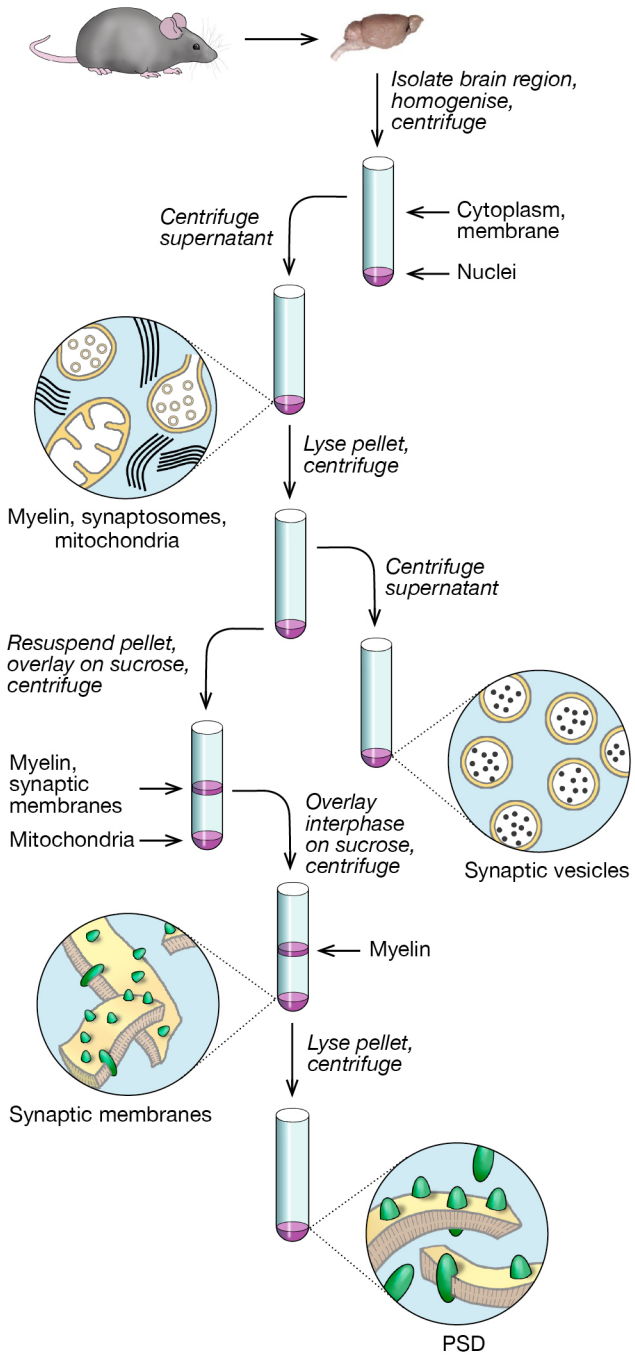

Fig. (4). Density gradient fractionation of neuronal tissue.

Phosphorylation is present at only low levels in cells and tissues of the nervous system. Sample preparation is a critical step in achieving the high-sensitivity, regional specificity and wide dynamic range needed by studies of phosphorylation. Most studies therefore involve sub-cellular fractionation of specific brain regions as the first step. Density gradient centrifugation is a well-established and reproducible method for the isolation of specific neuronal components such as the nucleus, synaptosomes, synaptic membranes, synaptic vesicles and the post-synaptic density.

QUALITATIVE ANALYSES OF PHOSPHORYLATION IN THE CENTRAL NERVOUS SYSTEM

Knowledge of the expression pattern and level of genes in distinct regions of the brain is well advanced (Lein *et al.*, 2007). However, such information regarding the quantities of proteins and their post-translational modifications has yet to be fully investigated or understood. Several MS-based studies have looked at the phosphoproteome of the brain (see Table **1**). Most have looked at sub-cellular fractions such as the PSD (Trinidad *et al.*, 2005; Trinidad *et al.*, 2006; Jaffe *et al.*, 2004; Munton *et al.*, 2007), or synaptosomes (Munton *et al.*, 2007; Collins *et al.*, 2005), from either mouse or rat, and in one case from human tissue (DeGiorgis *et al.*, 2005). In total these studies identified several thousand phosphorylation sites of which most were novel. Many of these sites were on proteins that play key roles in synaptic plasticity such as neurotransmitter receptors, protein phosphatases and kinases. These studies have highlighted the fact that protein phosphorylation in brain cells is widespread and is a critical regulatory mechanism of brain function.

The success of these studies has relied on the maximisation of the separation of peptides through the use of sub-cellular fractionation, orthogonal peptide separations and phosphopeptide enrichment techniques such as SCX-HPLC and IMAC. Additionally, increasing the overall level of PSD phosphorylation and potentially providing better coverage has also been employed (Jaffe *et al.*, 2004). However, in all studies most of the reported phosphoproteins are relatively abundant in the neuron, indicating further sub-fractionation is needed in future studies. Additionally, relatively few pTyr sites were identified, indicating that complementary enrichment methods such as anti-pTyr antibodies should be employed.

Another problem raised by these studies is that distinguishing between isoforms of proteins and between different proteins with a high degree of sequence similarity is difficult with the low sequence coverage typically observed in shotgun phosphoproteomic experiments (Nesvizhskii and Aebersold, 2005). This becomes a significant issue in phosphoproteomics as it may be impossible to assign the detected phosphopeptides to any single isoform or protein type. In such instances more targeted approaches are needed, such as the isolation of single proteins prior to digestion to unambiguously assign the site (John *et al.*, 2007).

The emphasis of these studies was primarily on the identification of phosphoproteins in basal conditions rather than on the analysis of changes in phosphorylation following activity, or their biological significance. Thus, most data available to date consists of lists of phosphorylation sites and proteins. While a hugely valuable resource for further studies, such lists don't help determine what is the most relevant to specific areas of neuroscience research. Consequently it is likely these resources will remain largely underutilised due to the huge amount of data researchers will need to wade through and the lack of functional annotation. Several curated web-based bioinformatics resources dedicated to aggregating information on protein phosphorylation exist, in-

Table 1. A Selected List of Phosphoproteomic Studies

		Labelling Method for Phosphopeptide Quantification					
		No quantification	Metabolic SILAC/SILAM	Enzymatic O^{18}	Chemical		
					Methyl-esterification/ propionylation	iTRAQ	ICAT
Phosphopeptide enrichment method	TiO_2	Bodenmiller *et al*., 2007a Macek *et al*., 2007 Larsen *et al*., 2005	Benschop *et al*., 2007 Dengjel *et al*., 2007				
	IMAC	Bodenmiller *et al*., 2007a **Ishihama *et al*., 2007** **Munton *et al*., 2007** Villen *et al*., 2007 **Trinidad *et al*., 2006** **Collins *et al*., 2005** **DeGiorgis *et al*., 2005** **Trinidad *et al*., 2005** **Jaffe *et al*., 2004**	Erba *et al*., 2007	Bonenfant *et al*., 2003	Garcia *et al*., 2006 Zheng *et al*., 2005	Schmelzle *et al*., 2006 Zhang *et al*., 2005	
	Antibody	Villen *et al*., 2007 Rush *et al*., 2005			Guo *et al*., 2007 Zheng *et al*., 2005	Schmelzle *et al*., 2006 Petti *et al*., 2005 Zhang *et al*., 2005	Theleman *et al*., 2005
	Chemical derivitisation	Bodenmiller *et al*., 2007a Oda *et al*., 2001			Guo *et al*., 2007 Tao *et al*., 2005		
	SCX	Macek *et al*., 2007 **Munton *et al*., 2007** Villen *et al*., 2007 Beausoleil *et al*., 2006 **Trinidad *et al*., 2006** **Trinidad *et al*., 2005** **Ballif *et al*., 2004** Beausoleil *et al*., 2004	Benschop *et al*., 2007			**Munton *et al*., 2007**	
	No enrichment		Park *et al*., 2006		**Jin *et al*., 2005**	**Grant *et al*., 2007**	

Selected articles using different techniques for the enrichment and quantification of phosphopeptides. Articles dealing with the analysis of primary neuronal tissue are indicated in bold.

cluding PhosphoSite (Hornbeck *et al.*, 2004), SwissProt, and Phospho.ELM (Diella *et al.*, 2004). Other databases such as the human reference protein database (HRPD) (Peri *et al.*, 2003) include a compendium of phosphorylation motifs, and Phosida (Olsen *et al.*, 2006) contains temporal phosphorylation data from cell stimulation time-course experiments. The extension of these databases to include data on functional analyses will tremendously increase their usefulness in the future.

QUANTITATIVE ANALYSES OF PHOSPHORYLATION IN THE CENTRAL NERVOUS SYSTEM

Quantitative proteomics has been increasingly applied in studies examining changes in protein levels in the brain (Grant *et al.*, 2007; Hu *et al.*, 2006; Li *et al.*, 2007; McClatchy *et al.*, 2007b; Olsen *et al.*, 2007). Some studies have utilised techniques such as AQUA to determine the absolute molar amount of synaptic proteins such as CaMKII (Cheng *et al.*, 2006), but very few have utilised these technologies for quantitative phosphoproteomic analyses (see Table **1**). To date most large-scale quantitative phosphoproteomic studies have concentrated on model systems typically using SILAC cultured cells stimulated, for example, with epidermal growth factor (EGF) (Olsen *et al.*, 2006). However, studies that have applied these techniques, demonstrate the influence mass spectrometry will have in the future.

One study reported the use of iTRAQ to quantify changes in the phosphorylation stoichiometry of pSer and pThr in mouse synaptosomes before and after stimulation with KCl (Munton *et al.*, 2007). Demonstrating the usefulness of iTRAQ's four mass-tags, synthetic peptides corresponding to the unphosphorylated and phosphorylated forms of the proteins CAMKIIα/β and GluR1 were generated and labelled with two iTRAQ mass-tags. By 'spiking' these labelled peptides into the sample, the authors were able to determine both the relative and absolute molar change in phosphorylation due to KCl at these sites, and also showed that the level of the protein itself remained unchanged. In addition, synthetic peptides corresponding to other sites were also used to

determine the phosphorylation stoichiometry of proteins such as Gprin1. Significantly, the power of the approach allowed the quantification of two phosphopeptides that were similar enough that it would have been difficult to discriminate between them using classical approaches.

An aim of neuroproteomics is the study of phosphorylation stoichiometry in tissues from animal models. A step in this direction was demonstrated in another iTRAQ-based study where changes in protein phosphorylation were examined in the spinal cord of a rat model of multiple sclerosis (Grant *et al.*, 2007). While phosphorylation was observed at six unique sites, only four animals were compared in a pairwise experiment, which was unfortunately not sufficient for statistical analyses. This study was limited by currently available labelling methods, which only allow a maximum of four samples to be compared in an MS analysis. In this respect, the statistical validation of quantitative proteomic experiments requires the data from several MS analyses to be merged. Such merging is not completely straightforward, requiring a common 'reference sample' to be included in every analysis, through which several datasets can be indirectly compared. But since only a subset of peptides is detected and quantified in a typical experiment, not all phospho-/proteins will overlap; meaning potentially interesting sites may be missed. To try and overcome this, MS analyses therefore needed to be repeated multiple times on the same samples to increase the overlap, placing large requirements on machine time. New labelling methods such as 8-plex iTRAQ and the recently released 10-plex ExacTag may potentially help to simplify such studies.

A more targeted approach has been the immunoprecipitation of specific phosphoproteins from brain homogenate. Using propionylation for stable isotope labelling, the effect of three different drugs on the phosphorylation of DARPP32 was successfully determined. By relating changes in the phosphorylation stoichiometry to the unphosphorylated form of the protein, several independent analyses could be compared (Jin *et al.*, 2005).

SILAC-based experiments in cell culture provide a powerful means to develop hypotheses-driven studies that can subsequently be tested *in vivo*. SILAC cultured cells expressing the voltage gated potassium channel 2.1 (KV2.1) and mass spectrometry were used to detect sixteen phosphorylation sites on the channel, seven of which were shown to be regulated by the protein phosphatase calcineurin (Park *et al.*, 2006). This quantitative data allowed targeted electrophysiology experiments to be carried out specifically on the regulated sites, demonstrating their role in the regulation of neuronal firing. As a proof of principle, SILAM a similar technique, was also successfully utilised to study changes in the synaptosomal proteome during post-natal development (McClatchy *et al.*, 2007b). While this initial study focused on changes in unphosphorylated proteins, the use of this approach in companion with phosphoproteomics could provide a compelling workflow in the future.

To allow different unlabelled brain samples to be compared, one suggested approach is the use of reference peptides termed CDITs (culture derived isotope tags). In this approach isotopically labelled peptides derived from cells grown in SILAC medium, are added to two different brain samples and act as a means to indirectly compare the two samples (Ishihama *et al.*, 2005). One drawback with this approach, however, is the lack of a true neuronal cell line with which to generate the reference peptides. In this study the neuron-like Neuro2A cell line was used; the peptides derived from it were reported to cover 97% of those found in the brain samples, however, complete coverage would be desirable.

These studies demonstrate what is now becoming possible using neuroproteomic techniques. The further application of quantitative phosphoproteomics will provide a powerful tool for elucidating brain biology in the future.

THE ANALYSIS OF EPIGENETIC MODIFICATIONS USING MASS SPECTROMETRY

An interesting application of quantitative phosphoproteomics will be the *in vivo* analysis in animal models of the impact of changes in the activity of key protein kinases and phosphatases (Genoux *et al.*, 2002; Isiegas *et al.*, 2006). A recent functional aspect of protein phosphorylation that is gaining momentum is its implication in the regulation of nuclear processes. In the nucleus, protein phosphorylation has important regulatory functions and many protein phosphatases are highly enriched and some are even exclusively expressed in the nucleus (Moorhead *et al.*, 2007). The post-translational modification of histone proteins by phosphorylation constitutes an important regulatory mechanism of gene expression. Together with acetylation, methylation, ubiquitination, and sumoylation, histone phosphorylation forms an epigenetic 'histone code' that is determined by the unique combination of these histone modifications and is unique for each gene (Strahl and Allis, 2000). In the brain, phosphorylation of histone H3 has been demonstrated to be involved in the formation of long term memory in the mouse (Chwang *et al.*, 2006). The use of mass spectrometry for the analyses of these modifications therefore has great appeal, and recently has begun to be applied to the analysis of histone variants and modifications (Garcia *et al.*, 2007b; Trelle and Jensen, 2007).

While the relative levels of phosphorylation at several sites on the histone H1 have been determined using stable isotope labelling and IMAC (Garcia *et al.*, 2006), such analyses are technically challenging. This is due to the small hydrophobic nature of histones, their high lysine content and the sequence similarity between histone isoforms, making peptide assignment difficult. The high lysine content prevents the use of trypsin (which cleaves after lysine and arginine) for protein digestion, as the resulting peptides are generally too small for MS analysis. Instead enzymes, such as AspN, that cleave after other amino acids have often been used, but these are typically less efficient and don't usually generate reproducible peptide fragments, essential for quantification. Some success, however, has been achieved with chemical derivitisation (e.g. propionylation) of the protein to mask the lysine residue and therefore allow the use of trypsin (Garcia *et al.*, 2007a; Syka *et al.*, 2004). All studies to date have analysed the phosphorylation state of histones extracted from bulk chromatin. However, as the methods for the mass spectrometry analysis of histones, including 'top-down' techniques (Siuti and Kelleher, 2007), become more mature,

it is likely that the phosphorylation state of individual genes can be assessed, providing a powerful tool in the future.

MAPPING PHOSPHORYLATION DATA ONTO SIGNALLING COMPLEXES

Quantitative proteomics has been applied to the study of multi-protein complexes, allowing the identification of many interaction partners (Ranish *et al.*, 2003; Gingras *et al.*, 2007). The major multi-protein complexes in the CNS that have been studied by proteomics to date are the NMDA receptor complex (Husi *et al.*, 2000), the PSD-95 complex (Husi and Grant, 2001), mGluR5 (Farr *et al.*, 2004), the MASC (MAGUK associated signalling complex) and ARC (AMPA receptor complex) (Collins *et al.*, 2006). In the PSD these complexes act as signal transduction 'machines', combining a diverse array of inputs and transmitting the signals through downstream pathways (Valor and Grant, 2007). Central to the functioning and formation of these signalling complexes is the process of reversible phosphorylation. Phosphorylation can alter protein function through conformational changes and by creating binding sites for protein-protein interactions. The feasibility of using quantitative MS for investigating phosphorylation dependent protein-protein interactions, was demonstrated in a recent study utilising iTRAQ (Zhou *et al.*, 2007). SILAC was used in a similar study involving the identification of signalling proteins, and their sites of phosphorylation, previously isolated as a result of their phosphospecific binding to other tyrosine-phosphorylated proteins (Hinsby *et al.*, 2004). Because the post-synaptic proteome comprises an array of macromolecular protein complexes, phosphoproteomic methods will prove useful in defining the role phosphorylation plays in the types of interactions in these complexes.

An important area for data mining will be the merging of existing datasets describing phosphorylation sites and dynamic phosphorylation time courses, with those describing the composition of multi-protein complexes. Several groups have started to apply phosphoproteomic data to signalling complex data gathered in other experiments, linking phosphorylation sites to specific kinases by way of consensus sequences and through proximity by their presence in specific multi-protein complexes (Collins *et al.*, 2005).

CONCLUSIONS

To date thousands of phosphorylation sites have been identified by discovery-driven phosphoproteome wide studies. This resource now provides a base-layer onto which subsequent research can be built. However, neuroproteomics is moving away from a purely technology development phase, typified by the discovery-driven generation of phosphosite lists. Instead, neuroproteomic techniques have matured to the point that the tools are now available to target specific phosphorylation pathways. New technologies, including electron transfer dissociation (ETD) (Mikesh *et al.*, 2006), multiple reaction monitoring (MRM) (Malmstrom *et al.*, 2007), should allow the neuroscience field to focus on specific sites and perform time course experiments that show the temporal dynamics of phosphorylation *in vivo*. Such experiments are already possible on a small scale using flow cytometry of single cells with validated phosphospecific antibodies (Perez and Nolan, 2006). These tools will hopefully be exploited and applied to the various animal models already available to elucidate the underlying mechanisms of neuronal signalling and brain functions. By extension, their application to pathological brain samples should help uncover the mechanisms of some diseases and may lead to the development of novel therapies.

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ABBREVIATIONS

AQUA	=	Absolute quantification
CNS	=	Central nervous system
Da	=	Dalton
HPLC	=	High performance liquid chromatography
IMAC	=	Immobilised metal affinity chromatography
iTRAQ	=	Isobaric tags for relative and absolute quantification
MS	=	Mass spectrometer
PAC	=	Phosphoramidate chemistry
PSD	=	Post-synaptic density
PTM	=	Post-translational modification
RP	=	Reversed phase
SCX	=	Strong cationic exchange
pSer/pThr/pTyr	=	Phosphoserine/phosphothreonine/phosphotyrosine
Ser/Thr/Tyr	=	Serine/threonine/tyrosine
SILAC	=	Stable isotope labelling of amino acids in cell culture
SILAM	=	Stable isotope labelling of amino acids in mammals
TiO_2	=	Titanium dioxide

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